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Production of protease by *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolated from abattoir environment

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ABSTRACT:

The study investigated the optimum conditions of temperature, pH, inoculum size and time of incubation on bacterial protease production. Protease producing bacterial species were isolated from abattoir soil and identified as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The optimum conditions observed for protease production was 37 ^{o}C at pH 9, with 2 % inoculum in the medium for 24 h of incubation. *Pseudomonas aeruginosa* had higher protease activities at optimum temperature and pH than *Staphylococcus aureus* while *S. aureus* had higher protease activities at optimum incubation time of 24 h and inoculum concentration of 2 %. The study gave evidence that these bacterial isolates could be potentially applied in biotechnological processes.

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INTRODUCTION

Proteases are the most important industrial enzymes that execute a wide variety of functions and have various important biotechnological applications (Mohen and Dilee, 2005). They constitute two thirds of the total enzymes used in various industries and account for at least a quarter of the total global enzymes production, which represent about 60 % of all the industrial enzyme's sales in the world, due to their applications in several industrial sectors (Kumar *et al.,* 2002; Gupta *et al*., 2002). Properties of this protease such as alkaline pH, thermo stability in solvents and detergent resistance make the enzyme useful for different applications. Proteolytic enzyme producers are also helpful for the health of the ecosystems of this earth as these microbes decompose the dead and decaying animal or plant tissues in water or land. They can create pollution free environment and they are responsible for the recycling of nutrients (Gupta *et al.*, 2007).

The induction of protease requires a substrate having peptide bonds including substrates like peptone, casein and other proteins. The ammonia, as final product of enzymatic reaction of substrate hydrolysis, represses enzyme synthesis by a well-known mechanism of catabolite repression. This extracellular protease has also been commercially exploited to assist protein degradation in various industrial processes (Srinubabu *et al.* 2007). The great advantages offered by microbial enzymes are low material costs coupled with high and faster productivity and the ease with which the enzymes can be modified (Sharma *et al.* 2007). At present, due to high cost of substrates and mediums used, the overall cost of enzyme production is very high and therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable from the commercial point of view (Kammoun *et al*. 2008).

Proteases show variety of characteristics under different conditions. Microorganisms, which produced extracellular acid proteases, often acidify the medium in which they grow (Shumi, *et al*., 2004), and the ability to produce alkaline proteinases has been correlated with growth of organisms at neutral to alkaline pH (Shumi, *et al*., 2004). Formation of proteinases varies in the presence of different carbon and nitrogen sources (Shumi *et al*., 2004), medium pH (Hossain *et al*, 1999.), and also the incubation temperature and time (Marzan *et al.,* 2004; Shumi *et al*., 2004). Heat stable alkaline proteases, reported by many workers (Thangam and Rajkumar, 2002), have potential for industrial use.

Proteases are complex multi-enzyme system which catalyzes the hydrolysis of amide bond in a protein molecular hence it has been used in the field of textile processing for degumming of silk and processing of wool (Ravel and Banerjee, 2003; Adinarayana *et al*., 2005). With the advent of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields, such as clinical, medicinal and analytical chemistry. To meet the current largely increased demand, studies on the cost-effective production of industrially important enzymes have become the need of this day.

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. Habitats that contain protein are the best sources to isolate proteolytic microorganisms. Waste products of meat, poultry and fish processing industries can supply a large amount of protein rich materials for bioconversion to recoverable products (Dalev, 1994).

The present study is aimed at the isolation of protease producing bacterial species from abattoir environment and possibly determines the optimum conditions for protease activity.

MATERIALS AND METHOD

Sample Collection and Isolation of Proteolytic Bacteria

The soil samples were collected from an abattoir environment in Owerri, Imo State, Nigeria. They were stored in ice and analyzed within one hour of collection. One gram of soil sample in a 250 ml flask was homogenized with 10 ml of sterile water; it was later made up to 100 ml with sterile water, mixed and shaken on a mechanical shaker for 45 minutes. An aliquot of the homogenized sample (0.2 ml) was spread on casein agar plates (nutrient agar supplemented with 36% casein) and incubated for 48 hours at 37 ^{0}C. The isolates were identified based on their morphological and biochemical characteristics (Holt *et al*. 1994).

Screening of Proteolytic Bacteria

Isolates were plated over nutrient agar medium that contains 0.4 % gelatin and incubated

at 37 ^{0}C for 24hrs. Plates were flooded with 1% tannic acid. Isolates having a higher zone of clearing were grown in liquid broth and stored for subsequent use.

Protease Activity Assay

The protease activity was determined according to the method of Anson (1938) with some modifications. The isolates were grown in a medium containing 10 g of glucose, 5 g of casein, 5 g of yeast extract, 2 g of KH_2 PO_4 and 10 g of Na_2CO_3 in 100 ml of sterile water. After sterilization, the medium was inoculated and incubated at 37 ^{0}C for 48 h. After incubation the culture filtrates were collected by centrifugation at 1000 x *g* for 12 minutes at 4^{0}C. The supernatant was used as crude enzyme. An aliquot (0.5 ml) of the crude extract was mixed with 5.0 ml of Tris-Hcl buffered casein and incubated at 37 ^{0}C for 30minutes. After incubation, 5 ml of 110 mM Trichloroacetic Acid (TCA) was added to stop the reaction. The mixture was centrifuged at 10,000 rpm for five minutes and the released amino acids were measured as tyrosine using the method of Folin and Ciocalteu, (1929) by calculating the amount of tyrosine in the extract using a tyrosine standard curve. The enzyme activity was expressed in units (U). One unit of enzyme was defined as the amount of enzyme that releases 1 µmol of tyrosine per ml of crude extract per minute.

Effect of Temperature on Protease Production

The effect of temperature on protease production was studied by incubating the culture media at different temperatures ranging from 27 ^{0}C-77 ^{0}C for 24 hrs. Protease activity was determined after 24 hrs of incubation.

Effect of pH on Protease Production

The effect of pH on protease production was determined by culturing the bacterium in the protease production media with different pH ranges (pH 5 to 11). The enzyme assay was carried out after 24 hrs of incubation at 37 ^{0}C.

Effect of Inoculum Concentration on Protease Production

Effect of Inoculum concentration on protease production was determined by inoculating the production medium with different concentrations (2-7 %) of overnight grown bacterial culture. The inoculated medium was incubated at 37 ^{0}C for 24 hrs after which the culture medium was centrifuged at 5000 rpm at 4 ^{0}C for 15 mins. The protease activity was determined as stated above.

Effect of Incubation Time on Protease Production

The effect of incubation time on protease production was determined by incubating the culture medium at 37 ^{o}C and at different time intervals (24-168 hrs) with samples taken for analysis at interval of 24 hrs. Protease activity was also determined as stated.

Statistical Analyses

Data obtained from this study were analyzed using a one-way analysis of variance (ANOVA) and values for $P \leq 0.05$ were considered statistically significant.

RESULT AND DISCUSSION

The study investigated the optimum conditions for protease production in *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The parameters investigated included the effect of temperature, pH, inoculum concentration and time of incubation on the production of protease. It was discovered that the parameters investigated had varying effects on the protease activities of the isolates.

Temperature had effect on the production of protease in both organisms. In *Pseudomonas aeruginosa*, there was an increase in protease production with increase in temperature up to the temperature of 37 ^{o}C. After this temperature, the protease production reduced a little with equal protease activity at 47^{o}C and 57 ^{o}C, reduced a little more at the temperature of 67 ^{o}C and reduced drastically at 77 ^{o}C. The highest protease production was observed at 37 ^{o}C, **(Fig. 1).** In *Staphylococcus aureus*, there was an increase in protease production with an increase in temperature up to the maximum at 37 ^{o}C. After this temperature, the protease production decreased with increase in temperature. Generally, temperature had significantly more effect on *Staphylococcus aureus* than *Pseudomonas aeruginosa* at $P \leq 0.05$. It was discovered that there was an increase in protease production with increase in temperature up to the maximum protease activities at the temperature of 37^{o}C in both organisms. Kalaiarasi and Sunitha (2009) also reported a similar trend in *Pseudomonas fluorescens* where they observed that the organism could produce protease in the range of 27-57 ^{o}C with production maximum at 37 ^{o}C. However, increase in temperature beyond 37 ^{o}C led to the declination of protease production proving that temperature plays a major role in enzyme production.

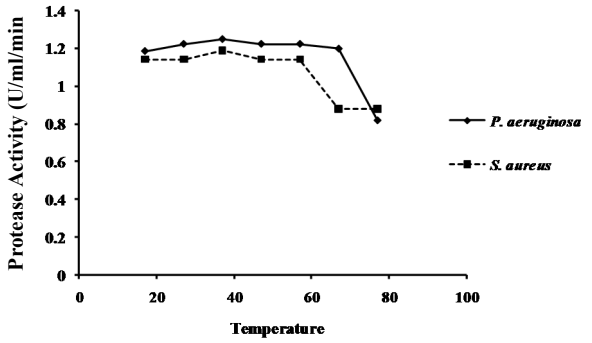

Fig. 1. Effect of temperature on protease activities of the isolates

The result showed that both organisms were affected by an increase in pH. In both organisms, protease production increased with increase in pH, **(Fig. 2).** Their maximum protease productions were at pH 9. However, at pH 9, *P. aeruginosa* had slightly more protease activity (1.24847 U/ml/min) than *S. aureus* (1.23587 U/ml/min). The lowest protease activities in both organisms were at pH 11. At pH 6 and 7, both organisms had equal protease activities (1.19804 U/ml/min). Statistical analysis showed no significant difference on the effect of pH on the protease activities of both organisms, ($P \leq 0.05$).

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Increase in pH resulted in corresponding increase in protease production up to the maximum protease production at pH 9 (1.24847 U/ml/mim and 1.23587 U/ml/min, respectively for *P. aeruginosa* and *S. aureus*). The result showed that there was a stimulation of the enzyme production at alkaline pH. The result obtained is in consonance with the work of Kumar *et al*. (2002) who reported that protease production was at maximum in pH 7 and 9 for *Bacillus* sp. and *Pseudomonas* sp. respectively. This same result was also obtained by Amara *et al*. (2009) and Sathees Kumar *et al*. (2011) who observed an optimum protease activity of 34 Unit/ml and 215.56 U/ml for *Geobacillus* sp and *Pseudomonas aeruginosa*, respectively at pH 9. This was however at variance with the work of Sally, (2000) who, using Azocoll as a protease substrate, observed highest specific protease activity of *Burkholderia* strain 2.2 N at pH 7.5.

Increase in inoculum concentration increased the protease activity in both organisms up to the maximum at 2% inoculum concentration. Increasing the inoculum concentration beyond 2% resulted in the decrease of protease activity of both organisms **(Fig. 3).** There was a very high significant difference in the effect of inoculum concentration on protease activities of both organisms at $P \leq 0.05$. *S. aureus* had significantly higher protease activities than *P. aeruginosa*.

Initial inoculum concentration influenced the production of protease in both organisms. It was observed that increase in inoculum concentration increased the protease activity in both organisms up to the maximum of 1.10496 U/ml/min and 1.19804 U/ml/min, respectively for *P. aeruginosa* and *S. aureus* at 2% inoculum concentration. These results were in accordance with Elibol *et al*. (2005) who reported that 2.5% inoculum level gives higher protease production.

The result of the effect of time of incubation for protease production showed that there was a drastic decrease in protease production with increase in the time of incubation in both organisms **(Fig. 4).** The highest protease activity observed in both organisms was at 24 hours of incubation. In *P. aeruginosa*, there was no further decrease in protease activity between 120 and 144 hours of incubation. After 144 hours of incubation, there was a very sharp decrease in protease production at 168 hours of incubation. In *S. aureus*, there was no further decrease in protease production from 96

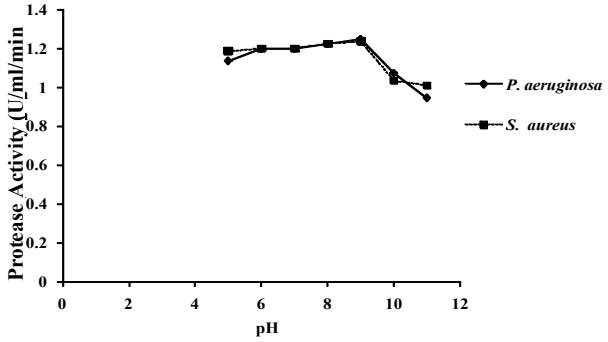

Fig. 2. Effect of pH on protease activities of the isolates

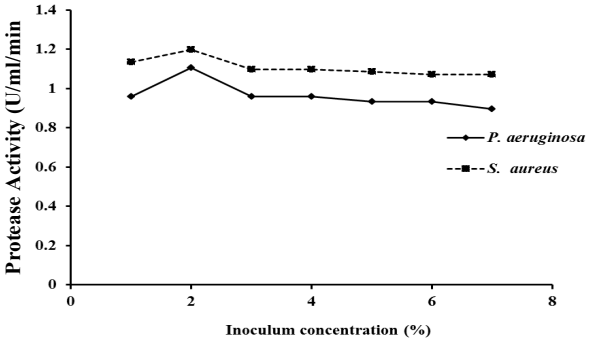

Fig. 3. Effect of inoculum concentration on protease activities of the isolates

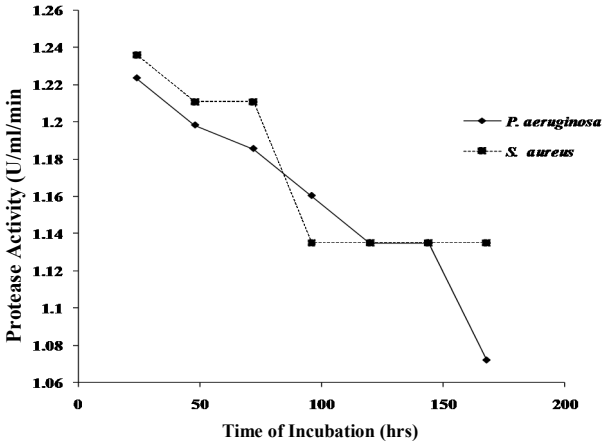

Fig. 4. Effect of time of incubation on protease activities of the isolates

hours of incubation till 168 hours of incubation. Both organisms had equal protease activities at 120 and 144 hours of incubation (1.13499 U/ml/min). However, there was no significant difference on the effect of incubation time on protease activities of both organisms, ($P \leq 0.05$).

The highest protease activity observed in both organisms was at 24 hours of incubation. This finding is in partial agreement with the work of Kumar *et al*. (2002) who reported that *Pseudomonas* sp. S22 showed a peak for protease production at 24 h of incubation and again at 108 h of incubation. However, the present result is in total agreement with the work of Kalaiarasi and Sunitha (2009) who observed the same peak protease production at 24 h of incubation.

The data gathered in this study has provided evidence for the protease producing ability of abattoir-soil-isolated *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The influence of environmental factors on the protease production of the isolates was also evident in this study as is being corroborated by some other authors (Tambekar and Tambekar, 2011). This study has given a hint that the microbial wealth of protease producing bacteria isolated from abattoir environment can be harnessed for biotechnological processes. The appreciable high enzyme activity at alkaline pH suggested that *P. aeruginosa* and *S. aureus* are potential producers of alkaline proteases which can find application in detergent and textile industries.

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